Evaluation of large volume yeast interfering RNA lure-and-kill ovitraps for attraction and control of *Aedes* mosquitoes

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Abstract. *Aedes* mosquitoes (Diptera: Culicidae)*,* principle vectors of several arboviruses, typically lay eggs in man-made water-filled containers located near human dwellings. Given the widespread emergence of insecticide resistance, stable and biofriendly alternatives for mosquito larviciding are needed. Laboratory studies have demonstrated that inactivated yeast interfering RNA tablets targeting key larval developmental genes can be used to facilitate effective larvicidal activity while also promoting selective gravid female oviposition behavior. Here we examined the efficacy of transferring this technology toward development of lure-and-kill ovitraps targeting *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse) female mosquitoes. Insectary, simulated field, and semi-field experiments demonstrated that two mosquito-specific yeast interfering RNA pesticides induce high levels of mortality among larvae of both species in treated large volume containers. Small-scale field trials conducted in Trinidad, West Indies demonstrated that large volume ovitrap containers baited with inactivated yeast tablets lure significantly more gravid females than traps containing only water and were highly attractive to both *A. aegypti* and *A. albopictus* females*.* These studies indicate that development of biorational yeast interfering RNA baited ovitraps may represent a new tool for control of *Aedes* mosquitoes, including deployment in existing lure-and-kill ovitrap technologies or traditional container larviciding programs.

Introduction

Dengue, a leading cause of morbidity in the tropics, Zika, chikungunya, as well as urban yellow fever, all result from infections with arboviruses primarily transmitted through the bites of *Aedes* females. *Aedes aegypti* is widely distributed in the subtropics and tropics, and is the primary arbovirus mosquito vector in Trinidad, West Indies (Rawlins *et al.*, 1998). A secondary arbovirus vector, *A. albopictus,* was first recorded in Trinidad in 2003, where it was identified in

the northwest region of the island (Chadee *et al.,* 2003). In Trinidad and Tobago, as well as other arboviral disease endemic countries, vector control is the primary means of preventing arboviral-induced diseases. The Insect Vector Control Division of the Trinidad Ministry of Health in Trinidad and Tobago currently targets *Aedes* mosquitoes through larval source reduction campaigns, focal larvicide treatments with *Bacillus thuringiensis israelensis* (Bti) and Aquatain AMF™, and less frequently, with ultra-low volume adulticides (malathion) (http:www.health.gov.tt). The female *A. aegypti* preference for man-made container breeding sites near human dwellings provides an opportunity for the surveillance and control of *Aedes* mosquitoes through the use of ovitraps, typically small dark colored containers filled with water and, sometimes, attractants to lure gravid females. Ovitraps are non-invasive, inexpensive, and easy to use, and have been deployed for mosquito surveillance in many countries afflicted by mosquito-borne illnesses, including Trinidad (Chadee *et al.,* 1993).

Ovitraps have recently been investigated as tools for effective mosquito population control and arboviral disease reduction. A broad range of ovitrap sizes, basic designs, and lethal control agents have been studied (Johnson *et al.,* 2017). These include simple traps with physical barrier screens that prevent any adults that develop from eggs deposited in them from escaping (Lok *et al.,* 1977), to more complex traps containing larvicidal agents (Perich *et al.,* 2003, Sithiprasasna *et al.,* 2003), as well as those with adulticidal activity (Mackay *et al.,* 2013, Eiras *et al.,* 2014). However, larger volume ovitraps baited with a hay infusion have been shown to be more attractive to gravid females and easier to maintain. The Gravid *Aedes* Trap (GAT) is a 10 L container baited with 3 L of water and hay infusion specifically designed to lure-and-kill female mosquitoes (Eiras *et al.,* 2014). The GAT typically employs an insecticide surface spray to kill individual mosquitoes that enter the trap, although alternative non-insecticide killing agents have been shown to be effective (Heringer *et al.,* 2016). The *Aedes* Gravid Trap (AGO) is a 19 L container baited with 10 L of water and hay infusion, and again, is designed to lure-and-kill female mosquitoes via inclusion of a sticky adhesive to capture and kill individual

4

mosquitoes once they enter the trap (Mackay *et al.,* 2013). Large-scale trials with AGO interventions in Puerto Rico have shown that urban *Aedes* populations can be significantly reduced, as well as the observed frequency of arbovirus infected females in the study populations (Barrera *et al.,* 2014, 2017).

Choice of effective lethal agents for control of *Aedes* and other mosquito vectors is complicated by many factors that often limit their useful application and success. Use of chemical insecticides can be ineffective due to rapid selection for and spread of resistance. Resistance to all commonly used broad spectrum insecticide classes has been reported among subtropical and tropical *Aedes* populations globally (Moyes *et al.,* 2017). Considerable progress has been made in developing more biofriendly and effective larvicides. These include insect juvenile hormone analogs (e.g., methoprene), bacterial derived toxins (e.g., Bti), and simple water surface films and surfactants (e.g., Aquatain) (Lawler 2017). However, even these still show some ecotoxicological effects on nontarget organisms likely to be found in mosquito breeding sites (Abe *et al.,* 2014, Lawler & Dritz, 2013, Vieira Santos *et al.,* 2017). Thus, it is important to continue development of new insecticides for controlling mosquitoes that further limit environment risks to nontarget organisms.

The RNA interference (RNAi) pathway is an endogenous antiviral response in mosquitoes and other organisms triggered by double stranded RNA (dsRNA) generated by RNA viruses during intracellular replication (Olson & Blair, 2015). The pathway is initiated by Dicer, which cuts longer dsRNA molecules into small interfering RNAs (siRNAs) that target and direct the silencing of genes with complementary sequences (Sontheimer, 2005). Interfering RNAs that target and silence essential mosquito genes could represent a novel class of pesticides with untapped potential for effective and targeted mosquito control. The short length (21-25 bp) of siRNAs, as well as their short hairpin RNA (shRNA) counterparts, allows for the effective design of interfering RNAs with conserved target sequences in multiple species of

vector mosquitoes that are not present in humans or other non-target species (Duman-Scheel, 2019).

We previously engineered *Saccharomyces cerevisiae* (baker's yeast) to produce shRNAs as larvicides that effectively target several different neural genes required for mosquito larval survival (Hapairai *et al.,* 2017, Mysore *et al.,* 2019a,b). Two-choice oviposition experiments conducted in the laboratory demonstrated that gravid *A. aegypti* females preferred to lay eggs in small volume (300 ml) containers bearing water baited with a yeast tablet rather than water alone (Hapairai *et al.,* 2017). The goal of the current investigation was to examine the efficacy for using yeast generated interfering RNA larvicides in larger volume ovitraps in which the yeast larvicides are expected to both attract gravid female mosquitoes to deposit eggs and subsequently kill the larvae that hatch from these eggs. We also examined attractiveness of yeast tablets as bait in larger volume containers in a field setting in Trinidad, West Indies.

Materials and methods

Mosquito strains and laboratory culturing

$A. aegypti$ and *A. albopictus* strains established from eggs collected in ovitraps in Trinidad, and F_2 larvae were assessed in this investigation. The *A. aegypti* Liverpool-IB12 (LVP-IB12) laboratory strain was used in several experiments. Mosquito strains were reared in an insectary at the Indiana University School of Medicine – South Bend, Indiana, U.S.A. following a standard protocol (Clemons *et al.,* 2010). Defibrinated sheep blood (HemoStat Laboratories, Dixon, CA) was provided as a blood source for females using an artificial membrane feeding system (Hemotek Limited, Blackburn, UK).

Yeast RNA larvicide tablet preparation

Stably transformed *Saccharomyces cerevisiae* control (Hapairai *et al.,* 2017), syt.427 and sema.460 strains were used for preparation of inactivated yeast interfering RNA larvicide tablets (~70 mg in size) as previously described (Mysore *et al.,* 2019c). syt.427 expresses shRNA that silences the *synaptotagmin (syt)* gene, and sema.460 expresses shRNA that silences the *semaphorin1a (sema1a)* gene, resulting in severe neural phenotypes. The 25 bp target sites of these larvicides are conserved in the *syt* and *sema1a* orthologs of multiple disease vector mosquitoes, including *A. aegypti* and *A. albopictus*, and have been shown to induce high rates of larval mortality in both species in small volume (500 ml with 100 ml distilled water) containers; these target sites have not yet been identified outside of mosquitoes and were not found to be toxic to select non-target arthropods that consumed the yeast larvicides in laboratory trials (Mysore *et al.*, 2019a,b).

Insectary trial of larval mortality

Yeast interfering RNA larvicide assays were conducted using previously described methods (WHO, 2005; Mysore *et al.,* 2019c) in 7.5 L plastic containers (diameter = 21 cm, height = 24 cm), filled with 3.5 L of water (water depth = 10 cm). Twenty newly hatched first-instar larvae and a single yeast tablet (control, syt.427, or sema.460) were added to each container. Twelve independent biological replicates were performed in an insectary at the Indiana University School of Medicine – South Bend with the *A. aegypti* Trinidad strain and *A. albopictus* Trinidad strain, respectively. Insectary conditions were 26° C, 84% relative humidity, with a 16 hr light and 8 hr dark cycle (Clemons *et al.,* 2010).

Incubator simulated field larvicide trials

Seven biological replicates with 20 newly hatched first-instar *A. aegypti* LVP-IB12 strain larvae per assay were performed in 7.5 L plastic containers filled with 3.5 L of water and a single yeast larvicide tablet in an incubator under similar environmental conditions as

the insectary, but programmed to better simulate temperature fluctuation under tropical conditions, with daytime temperature set at 33° C and nighttime temperature set at 26° C. The observed proportions of mortality were arcsine transformed and subjected to ANOVA followed by Tukey's HSD post-hoc comparison tests.

Semi-field larvicide trials

Semi-field trials were conducted following WHO larvicide testing guidelines (WHO, 2005) using *A. aegypti* LVP-IB12 larvae in screened enclosures on an outdoor roof top laboratory of the Galvin Life Sciences building at the University of Notre Dame, Indiana, U.S.A. as described elsewhere (Mysore 2019a,b). Twelve independent biological replicates with 20 newly hatched first-instar larvae per assay were conducted with 7.5 L plastic containers bearing 3.5 L of distilled water. These studies were performed in July – August 2018, at which time roof top ambient temperatures ranged from 13.5° C-42.0° C. The mean daytime temperature was 27° C, and the mean nighttime temperature was 23° C. Larval mortality data were evaluated as described above.

Comparison of hay infusion and Bti to yeast larvicide oviposition attractiveness

Independent pairwise oviposition trials were conducted to compare the attractiveness of: i) inactivated control interfering RNA yeast-treated water vs. hay infusion-treated water, and ii) Mosquito Bits-treated water (Summit Responsible Solutions, Baltimore, MD; active ingredient = Bti*)* vs. inactivated control interfering RNA yeast-treated water. Yeast was prepared and used in the trials, which were conducted as previously described (Hapairai et al., 2017). Briefly, one gravid Trinidad strain female was released in a 30 cm x 30 cm x cm cage containing a 500 ml plastic cup containing 100 ml of distilled water and lined with a white paper towel. Hay infusion was prepared as described by Reiter et al. (1991), then strained and diluted to 10% as described by Chadee et al. (1993).

Mosquito Bits were purchased from Amazon and used according to the manufacturer's dosage instructions. Egg count data for yeast-treated vs. hay infusion-treated assays and for yeast-treated vs. Bti-treated assays were evaluated using a paired t-test (n=20 replicates and n=27 replicates, respectively).

Insectary trial of oviposition and larval mortality

Single gravid adult female *A. aegypti* LVP-IB12 mosquitoes were released into 30 x 30 x 30 cm cages containing a 7.5 L ovitrap placed in the insectary. The ovitraps were initially treated with five ~350 mg control, syt.427, or sema.460 inactivated yeast tablets per ovitrap. Females were allowed to deposit eggs on a white paper towel lining the entire inner circumference of the ovitrap. Following oviposition, the egg papers were removed, the eggs were counted, and the papers then reinserted in the ovitrap. An additional five yeast tablets were added to each container after one week. Ovitraps were monitored until all surviving larvae (counting initiated at L2) had developed into pupae. Six biological replicates were performed. Egg count and hatching data were subjected to ANOVA followed by Tukey's HSD post-hoc comparison tests. Larval mortality data were evaluated as described above.

Oviposition attractiveness field trials

Relative attractiveness of gravid females to yeast-treated vs. water-only containing ovitraps was assessed during 2018 in small-scale field studies conducted on the University of the West Indies (UWI) St. Augustine campus, Trinidad, West Indies (10°38'31.00" N 61°24'01.00 W, Figure 1a). The ~58 hectare campus includes 103 main buildings, including dormitory and food service halls, classroom, office, and laboratory buildings, a library, and multiple annex buildings. Outdoor athletic facilities, including cricket ovals, two soccer fields, a rugby field, tennis courts, as well as a cocoa research plantation, are also located on campus. Google Earth software was used to create maps of the study area.

Typically, the dry season occurs between the months of December to April, and the wet season during the months of May to November. The studies were designed to cover the wet season when mosquito indices are expected to be high (Chadee *et al.*, 2007). In 2018, unusually high precipitation levels were observed across the entire island, with rain continuing through December and many areas, including St. Augustine, experienced severe flooding during October.

Yeast-baited ovitraps were evaluated using paired ovitraps containing water-only (control) or water containing one ~70 mg inactivated yeast interfering RNA tablet per container. Yeast expressing control shRNA with no known target in mosquitoes or other organisms (Hapairai *et al.,* 2017) was used in these studies as the experiments were not designed to test for larvicidal activity. Studies were conducted from June 18 through December 14, 2018. Dark blue 10 L plastic buckets (top diameter 26 cm, height 26.5 cm, bottom diameter 21.5 cm) were used. Each ovitrap was set up with ~3.5 L of dechlorinated tap water (10.5 cm depth), the perimeter lined with a 73x10 cm germination seed paper (Anchor Paper Company, Saint Paul, MN), and then covered with a 10 mm mesh wire screen (Figure 1b). Each ovitrap contained a 6 mm diameter overflow hole in the side of the container.

Ovitrap pairs were positioned in shaded locations (Figure 1c) protected from wind, rainfall and direct sunlight (i.e. eaves, patio corners, garage corners, etc.) (Rawlins *et al.,* 1998, Chadee and Ritchie, 2010). Heat inactivated yeast pellets were prepared from the control strain as described above, and a single tablet was placed in each yeast-treatment ovitrap. During weekly servicing of the ovitraps, egg papers, water, and yeast were replaced after each ovitrap had been thoroughly rinsed with fresh water. Ovitraps were also permutated within the same location when serviced to account for location bias in the pairwise comparisons. After egg papers were collected from the ovitraps, all mosquito eggs were counted in the UWI insectary. While *A. aegypti* has historically been the

predominant dengue vector mosquito in Trinidad, *Aedes albopictus* has been reported in Trinidad since 2003 (Chadee *et al.,* 2003). To assess potential oviposition activity of both species, pooled samples of individual egg papers (combined control and yeast treatments) collected weekly during these experiments were hatched and reared to adulthood following a standard protocol (Clemons *et al.,* 2010). *Aedes* species identification was performed as described by Darsie (1981).

To evaluate oviposition preference for yeast-treated vs. water-only ovitraps, we used a Mixed Effects Negative Binomial count model conditional on the unobserved number of egg laying events in a given week for a given pair of traps. The unobserved number of egg laying events are treated as model parameters under the assumption of a first order autoregressive process. This model allows us to account for the autocorrelation in the time series data at each study site and to estimate the preference for the yeast-treated vs. water-only ovitraps at each site. Therein, we let $Y_{w,d,i}$ be the count of eggs in either the treatment ($d = 1$) or control ($d = 0$) trap in the ith pair in the wth week. That is, $Y_{w,0,i} \sim$ NegBin($50\ U_{w,i}\ \rho_i,\ \theta$) and $Y_{w,1,i} \sim$ NegBin($50\ U_{w,i}\ (1 - \rho_i),\ \theta$) where $U_{w,i}$ is the unobserved number of egg laying events (assuming an average of 50 eggs laid per event) in the ith pair for the wth week; ρ_i is the preference for the water-only trap in the ith ovitrap pair. We used the Gamma-Poisson parameterization of the Negative Binomial such that NegBin($\mu,\ \theta$) has mean μ and variance $\mu + \frac{\mu^2}{\theta}$ such that the parameter θ controls the variance in the number of eggs laid per event. The unobserved data are treated as inferred parameters with density $\sum_i \sum_{m=2}^{26} Normal(U_{m-1,i},\ \sigma)$. We also assumed that the preference parameter is Beta distributed, $\rho \sim$ Beta(a, b). The parameters $\sigma, a, b, \rho_i, U_{w,i}$ were estimated using Stan v2.24.0 (Stan Development Team, 2020). The variance in the number of eggs laid at each laying event (i.e. the value of θ in the model) cannot be estimated from the data and must be fixed. We fixed $\theta = 5$, which means that 95% of egg laying events will involve between 14 and 105 eggs. Based on this model we define the P-

value for the test that mosquitoes prefer the water-only traps as $\int_{0.5}^{1} \text{Beta}(\hat{a}, \hat{b})$ where $\hat{a}$ and $\hat{b}$ are the maximum likelihood estimates of a and b.

Ethics approval and consent to participate

Permission to conduct the field studies in Trinidad and Tobago was approved by the Southwest Regional Health Authority, a division of the Trinidad and Tobago Ministry of Health, as well as from the chair of the Department of Life Sciences at UWI. Although no human subjects were involved in these studies, building managers working near areas in which traps were installed were contacted regarding the nature and duration of the experiments. Ovitraps were clearly marked with a sticker indicating that the devices were part of a mosquito research study and should not be disturbed, as well as the contact information for Dr. Azad Mohammed at UWI, who served as the local point of contact for these experiments.

Results

Yeast larvicide activity in 7.5 L ovitraps

Controlled climate insectary studies with 20 first instar larvae per trial demonstrated that syt.427 and syt.460 were highly effective as larvicides in 7.5 L ovitraps against both *A. aegypti* and *A. albopictus* F$_2$ populations derived from Trinidad (Table 1). With *A. aegypti*, ~91-92% larval mortality was observed with both yeast larvicides compared to 5.4% with the yeast control (ANOVA, $F_{2,33}$ = 260.4; P<0.0001). With *A. albopictus*, ~89-94% larval mortality was observed with both yeast larvicides compared to 7.5% with the yeast control (ANOVA, $F_{2,33}$ = 289.5; P<0.0001).

It is possible that higher temperatures encountered in the tropics, which increase

larval growth rates and could potentially impact the stability of the yeast larvicides, may impact larvicide activity. Results from incubator simulated field studies with *A. aegypti* LVP-IB12 strain, where ambient temperatures varied from 33° C daytime to 26° C nighttime to better approximate tropical conditions, were similar to those from the insectary studies (Table 1), suggesting that larvicidal activities of the two yeast strains were independent of temperature regime. Larval mortality varied from ~86-91% with the yeast larvicides compared with 5.7% with the yeast control (ANOVA, $F_{2,18}$ = 219.1; P<0.0001).

Results from semi-field trials conducted in outdoor screened enclosures in Indiana further confirmed that larvicidal activities of the two yeast strains were independent of the highly variable environmental conditions during July – August 2018, with ambient temperatures ranging from 13.5 – 42.0° C (Table 1). Larval mortality varied from ~90-91% with the yeast larvicides versus 9.6% with the yeast control (ANOVA, $F_{2,33}$ = 314.7; P<0.0001).

Oviposition and larval mortality in 7.5 L ovitraps.

Gravid *A. aegypti* LVP-IB12 females released individually in indoor cages containing 7.5 L ovitraps that were treated with control, syt.427, or sema.460 yeast laid similar numbers of eggs, varying from ~110-113 eggs per female (Table 2, P>0.05). Larval hatch rates were also very similar across treatments, varying from ~92-95% (P>0.05). Significant larval mortality was observed in the syt.427 (83.4±3.12%) and sema.426 (86.2±2.3%) yeast interfering RNA ovitraps compared with only 3.0+1.4% in those baited with control yeast (Table 2, ANOVA, $F_{2,15}$ = 155.6; P<0.001). No significant differences were observed between the syt.427 and sema.460 yeast larvicide treatments (P>0.05).

Yeast oviposition attractiveness relative to hay infusion and Bti

Results from insectary studies confirmed that gravid females showed no significant

13

preference (P<0.05) for oviposition in 300 ml containers with control yeast (63.2$\pm$12.61) compared to hay infusion (38.18.96). Similarly, no significant preference (P<0.05) was observed with control yeast (46.7$\pm$3.71) compared to Bti (46.3$\pm$3.58).

Oviposition attractant field studies

The mean number of eggs collected from 26 weeks of pairwise sampling (n = 20 pairs) of 10 L ovitrap attractiveness during June through December 2018 are shown in Fig. 2 (the raw data are shown in Supporting information Fig. S1). Variability in oviposition rates per week largely coincided with the typical rainy season in Trinidad (Chadee *et al.,* 2007), with the exception that unusually high levels of precipitation were observed across the island during October. Adults reared from pooled samples of individual egg papers (combined control and yeast treatments) each week (with the exception of week nine where no adult data was obtained due to an earthquake on August 21 that disrupted insectary activities) were a mixture of *A. aegypti* and *A. albopictus*, with *A. aegypti* predominant early in the season and *A. albopictus* predominant later in the season.

Overall, a total of 13,217 eggs were deposited in the water-only control ovitraps, while a total of 28,005 eggs were deposited in the yeast-baited treatment ovitraps (32% in water-only ovitraps). The average site-specific preference for the water-only ovitrap, ρ_i, ranged from 9% to 39%. The females clearly had a significant preference for the yeast-baited traps (P=0.02). Fig. 3 shows both the point estimates of the average site-specific preference parameters and the inferred population distribution used to compute the reported P-value. The confidence intervals for the site-specific parameters are shown in Supporting information Fig. S1. The difference in the empirical proportion of eggs layed in water-only traps (32%) and the model-estimated mean preference for water-only traps (24%) is driven by a relatively small number of transient spikes in eggs laid in water-only

ovitraps in experimental sites where the average preference was clearly for yeast-treated

ovitraps (e.g. pairs 1, 7 and 14 in Supporting information Fig. S2). The model correctly

downweighs the relevance of those outlaying data by allowing additional heterogeneity in

the count process.

Discussion

We conducted a series of laboratory, semi-field and field studies to test the hypothesis

that yeast interfering RNA larvicide tablets provide effective biofriendly agents for attraction of

gravid *Aedes* spp. females and control of breeding success using larger volume ovitraps that

have shown promise in arbovirus control (Johnson *et al.,* 2017). The results demonstrated that

yeast interfering RNA larvicides syt.427 and sema.460, which have target sites that are

conserved in the *syt* and *sema1a* neural genes of both *A. aegypti* and *A. albopictus,* but not

nontarget organisms (Mysore *et al.*, 2019a,b), functioned well in simulated deployment studies

with large volume ovitraps (Table 1), including studies in which oviposition attraction and

larvicidal activities of the yeast interfering RNA larvicides were combined (Table 2). In addition,

pairwise field studies conducted in Trinidad demonstrated that large 10 L ovitrap containers

filled with 3.5 L of water baited with yeast interfering RNA tablets were significantly more

attractive than unbaited ovitraps to gravid *A. aegypti* and *A. albopictus* females (Tables 3, 4, Fig.

1). The preference for ovitraps baited with yeast interfering RNA tablets was evident throughout

the rainy season in Trinidad in 2018 (Fig. 2) during which mosquito breeding activity and

arbovirus transmission are typically highest (Chadee *et al.,* 2007).

Both *A. aegypti* and *A. albopictus* eggs were collected in the 10 L yeast-baited

ovitraps in Trinidad demonstrating the additional value of these traps for *Aedes*

surveillance. Although *A. albopictus* was previously reported in Trinidad (Chadee *et al.,*

2003), this is the first record of its presence in the St. Augustine locality. The abundance of

A. albopictus eggs laid in the ovitraps suggests that this vector is widespread in St. Augustine, and likely, is present throughout Trinidad. This is of particular interest due to the implication of having an additional arboviral vector species on disease transmission dynamics and vector control in Trinidad. Moreover, the abundance of both *A. aegypti* and *A. albopictus* eggs collected in Trinidad indicate that it will be critical to deploy yeast larvicides which are capable of targeting both species. Yeast interfering RNA larvicides syt.427 and sema.460 induced high levels of mortality of Trinidad field strains of *A. aegypti* and *A. albopictus* in 7.5 L ovitrap containers, which is comparable to results observed in both small (500 ml with 50 ml water) and large (30 L with 26 L of water) containers (Mysore *et al.*, 2019a,b), providing further evidence that yeast interfering RNA larvicides function well in a variety of container types, and that either larvicide could be deployed in the field in larger volume ovitraps.

The present investigation included simulated field trials that combined, for the first time, both the lure and the kill capacities of the yeast. Yeast larvicides syt.427 and sema.460 performed equally well in these trials, in which adult females deposited eggs in yeast-treated containers, after which the eggs were subsequently permitted to hatch in the yeast larvicide-treated water. The mean numbers of eggs collected per 7.5 L ovitrap in these simulations (n = ~110-113, Table 2) was larger than the mean number of eggs per positive 10 L yeast-baited ovitrap per week in the Trinidad field trials (n = 75.5), suggesting that the results obtained in these simulations are relevant to field applications. Effective killing of these larvae was observed after scaling the dose of yeast tablets provided from one 70 mg tablet for 20 larvae (used in initial trials reported in Table 1) to five 70 mg tablets in the lure-and-kill studies (Table 2). Although the average numbers of eggs laid in these simulations and in field studies were comparable, a maximum of 645 eggs was collected in a single 10 L ovitrap in a one week period in Trinidad, indicating that for practical implementation, yeast interfering RNA larvicide formulations will need to take such information into consideration. Further, given that the residual activity of the present yeast interfering RNA larvicide tablets is ~10

days in water (Hapairai *et al.,* 2017), new formulations will also need to take longer-lasting

stability into account as well.   Although it will be ideal if these new formulations are capable

of inducing 100% mortality over extended periods, in the laboratory, existing formulations

induce 100% mortality in every replicate container only when the larvae are reared as

individuals (Mysore et al., 2019a,b). These findings suggest that some larvae prefer to eat

dead/dying larvae rather than yeast, a behavioral phenomenon which will need to be further

assessed in the field.

Observations from this investigation are in alignment with the results of Eiras *et al.*

(2014) and Mackay *et al.* (2013), who demonstrated that larger volume ovitraps effectively

lure gravid females seeking suitable oviposition sites and provide further support for their

conclusions. Preliminary field trials demonstrated that the attractive capacity of the yeast

larvicides is not observed in smaller 0.5 L ovitraps (Hapairai et al, unpublished),

suggesting that the use of large 10 L yeast-treated ovitrap containers will be more

effective for mosquito control, Previous studies indicated that yeast interfering RNA

larvicides designed to kill mosquitoes pose little if any threat to non-target organisms

(Mysore et al., 2019a,b), suggesting that yeast interfering RNA baited larger volume

ovitraps could be developed and deployed as a new biorational mosquito control

intervention. Further, as the existing AGO and GAT lure-and-kill ovitraps are specifically

designed to target and prevent adult females from laying eggs in the traps,

recommendations have also been made to treat the hay infusions with biofriendly

larvicides to prevent adult development from eggs dropped into infusion (Johnson et al.

2017). Our insectary trials showed that yeast interfering RNA larvicides, like hay

infusions, promote oviposition behavior, and mosquitoes showed no preference for

laying eggs in yeast-treated containers vs. containers treated with a commercial Bti

formulation, suggesting that the yeast larvicides could easily be incorporated in the use

of these or other traps, or in traditional larviciding programs. Resistance to every major

class of pesticides has been observed in mosquitoes across the globe, highlighting the

importance of developing new classes of insecticides, such as RNAi-based pesticides, for

mosquito control (Airs and Bartholomay, 2017). Evidence of resistance to the yeast

larvicides has yet to be encountered in ongoing studies, but will need to be further

monitored. The continued development of new strains of yeast larvicides, including strains

that express interfering RNAs that target multiple genes, will help to combat resistance that

emerges to any specific interfering RNA molecule,

  As discussed elsewhere (Duman-Scheel, 2019), the development and evaluation

of long-lasting larvicide formulations with several months of residual activity is ongoing.

Long-lasting Bti formulations, which are active in the field for five months, are emerging

as a promising control method for malaria vector mosquitoes (Derua et al., 2018), and it

is anticipated that similar long-lasting yeast larvicide formulations will greatly improve

the efficacy of lure-and-kill ovitraps that target *Aedes* mosquitoes. Such formulations

would be ideal for user-friendly ovitrap applications given that ovitrap maintenance is

required, and a lack of ovitrap maintenance can convert them into mosquito breeding

sites (Ritchie et al., 2008).

Data availability statement

The data that support the findings of this study are available from the corresponding author

upon reasonable request.

encouragement will have a lasting impact on our continuing research. We thank Nicole Achee and John Grieco for productive discussions about study design and are grateful to David Kang, Nikhella Winter, and Akilah Stewart for their advice and technical assistance. We also thank Professor Scott Ritchie for advice on selecting ovitraps. This work was funded by U.S. Department of Defense Awards W81XWH-17-1-0294 to MDS and W81XWH-17-1-0295 to DWS, as well as NIH/NIAID Award 1 R21 AI128116-01 to MDS and DWS. The funding agencies did not impact the experimental design or the decision to publish these data.

Author contributions

LKH, KM, AM, MDS, and DWS contributed to design and coordination of the study. LKH, LDJ, and RSF carried out field studies in Trinidad. LKH, KM, NDS, JSR, LS, and LEG contributed to the laboratory studies. NDS and KM conducted the semi-field trials. LKH, KM, ERS, and DWS analyzed the data. LKH, KM, ERS, AM, MDS, and DWS drafted and revised the manuscript. All authors read and approved the final manuscript.

Conflict of Interest Statement

MDS and DWS are inventors on pending International Patent Application (U.S. No.: 62/361,704, European No. 17828458.4). This application did not impact their interpretation of the data in this study, nor will it impact the authors' adherence to journal policies on sharing materials and data. All other authors declare no conflict of interests.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1: Weekly counts from treated and water-only ovitraps. Counts of eggs laid per week in treated (blue) and water-only (red) stratified by collection site.

Figure S2: Confidence intervals on site-specific preference parameters. The point estimates (black dot) and both 50% CI (red bar) and 95% CI (black line) are shown for each of the collection sites. The plotted value corresponds to the model parameter ρ_i (water-only preference).

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Figure Legends

Fig. 1. Assessment of gravid *Aedes* female attraction to yeast-baited ovitraps. (a) Map of Trinidad showing location of University of West Indies campus (star) in St. Augustine; (b) 10 L ovitraps; (c) locations of paired ovitraps (stars) on the University of West Indies campus; (d) total number of mosquito eggs collected by each trap pair with relative distribution between water-only control and yeast-baited traps.

Fig. 2. Weekly egg collection (mean±SE) of water-only control and yeast-baited 10 L ovitraps, and relative proportions of *Aedes aegypti* and *Aedes albopictus*. Rainfall (mm) data from Piarco airport (~8 km from UWI) are included for general reference. *No data collected due to disruption caused by an earthquake.

Fig. 3. Population density and point estimates of preference for the yeast-baited ovitrap in each experimental pair. Black circles indicate the point estimates of the preference for the treated trap

at each experimental pair, $\rho_{d,i}$. The plotted density shows the population density of preference

parameters, Beta(3.5, 10.9). The black dashed line shows the population mean and the red

dotted lines show the 95% confidence interval for the distribution.

Table Legends

Table 1. Larval mortality in 7.5 L ovitrap assays under laboratory and semi-field conditions.

Table 2. Oviposition by individual *A. aegypti* females and larval mortality in 7.5 L ovitraps

under laboratory conditions.

Table 1. Larval mortality in 7.5 L ovitrap assays under laboratory and semi-field conditions.

Assay/species, strain	% Larval mortality ($\pm$SE)			
	Control	syt.427	sema.460	F (Df)
Insectary				
A. aegypti, Trinidad	5.4 (2.17)[a]	91.7 (5.37)	91.3 (1.25)	260.4 (2,33)[b]
A. albopictus, Trinidad	7.5 (1.44)[a]	89.2 (1.61)	93.8 (1.52)	289.5 (2,33)[b]
Incubator simulated field				
A. aegypti, LVP-IB12	5.7 (2.02)[a]	86.4 (2.37)	90.7 (0.71)	219.1 (2,18)[b]
Semi-field				
A. aegypti, LVP-IB12	9.6 (1.99)[a]	90.0 (1.07)	92.1 (1.77)	314.7 (2,33)[b]

[a]Results in the control group (water-only) are significantly different (ANOVA with Tukey's HSD, $P<0.05$) than syt.427 or sema.460 groups. [b]$P<0.0001$.

Table 2. Oviposition by individual *A. aegypti* females and larval mortality in 7.5 L ovitraps under laboratory conditions.

Variable	Mean (SE)			F (Df)	P
	Control	syt.427	sema.460		
Number of eggs	112 (4.89)	110 (4.72)	113 (5.34)	0.148 (2,15)	0.86
Egg hatch rate (%)	92.1 (2.35)	95.2 (1.67)	95.4 (1.63)	0.584 (2,15)	0.57
Larval mortality (%)	3.0 (1.38)[a]	83.4 (3.12)	86.2 (2.3)	155.6 (2,15)	<0.001

[a]Results in the control group (water-only) are significantly different (ANOVA with Tukey's HSD, P<0.05) than syt.427 or sema.460 groups.

A

B

C

D

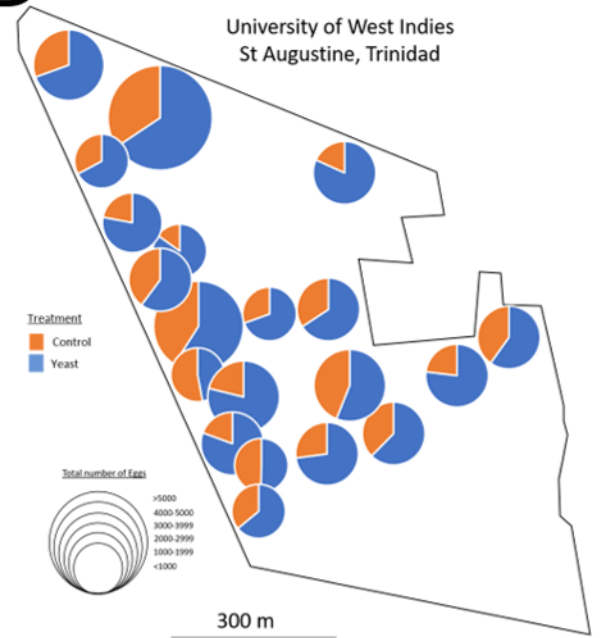University of West Indies
St Augustine, Trinidad

Treatment
Control
Yeast

Total number of Eggs
>5000
4000-5000
3000-3999
2000-2999
1000-1999
<1000

300 m
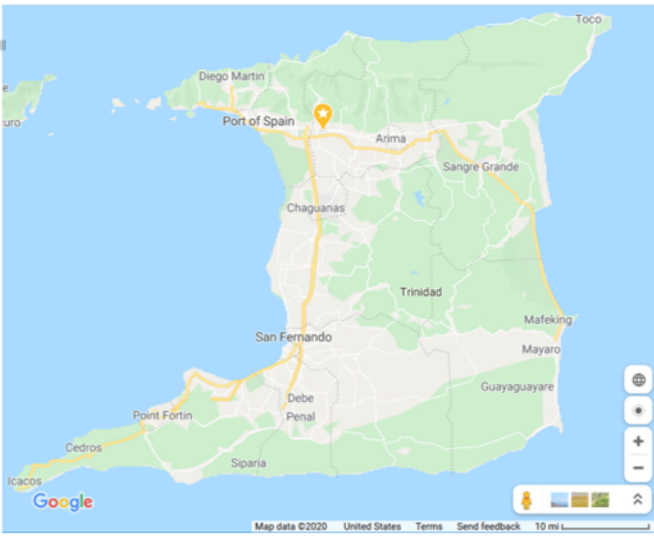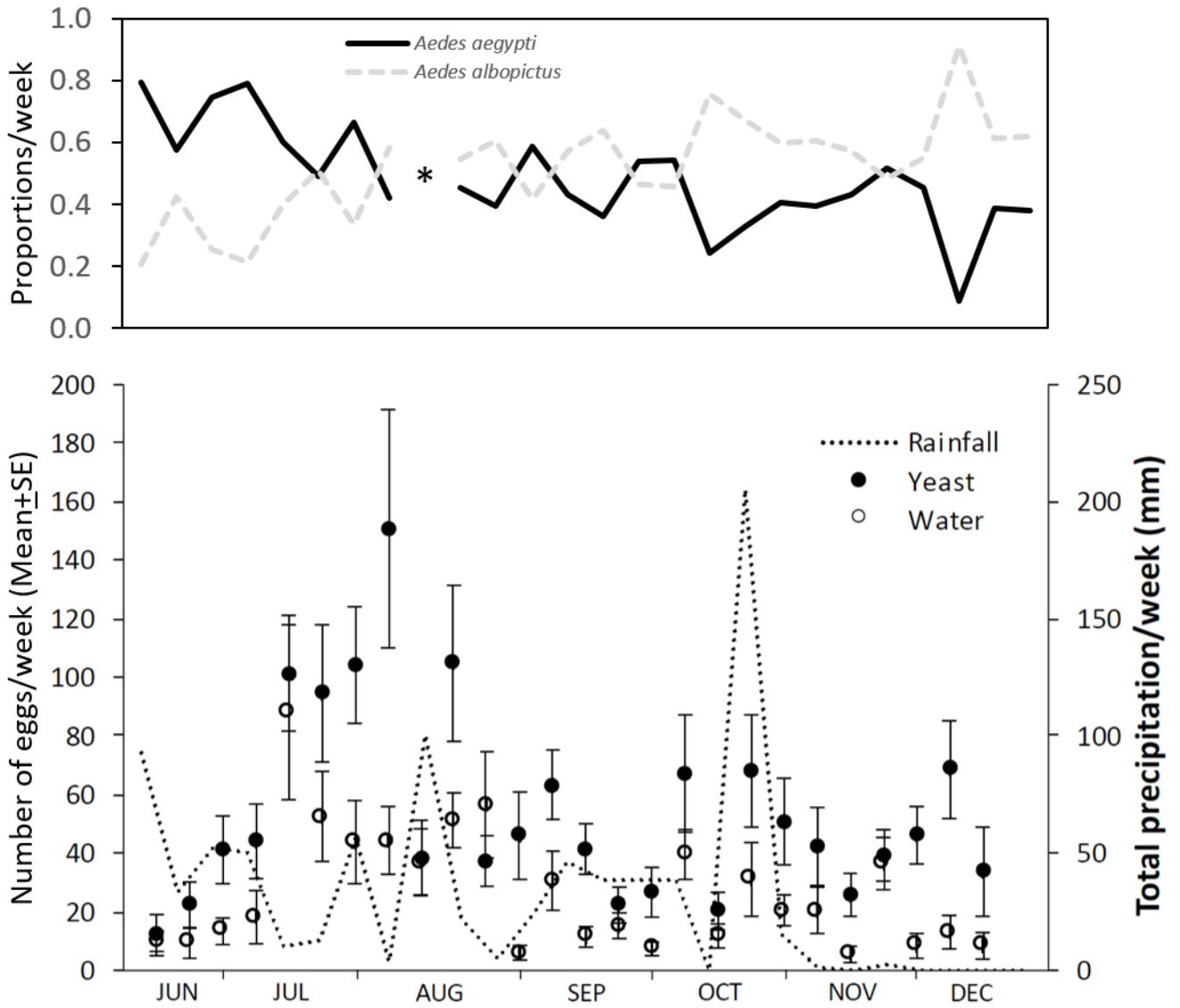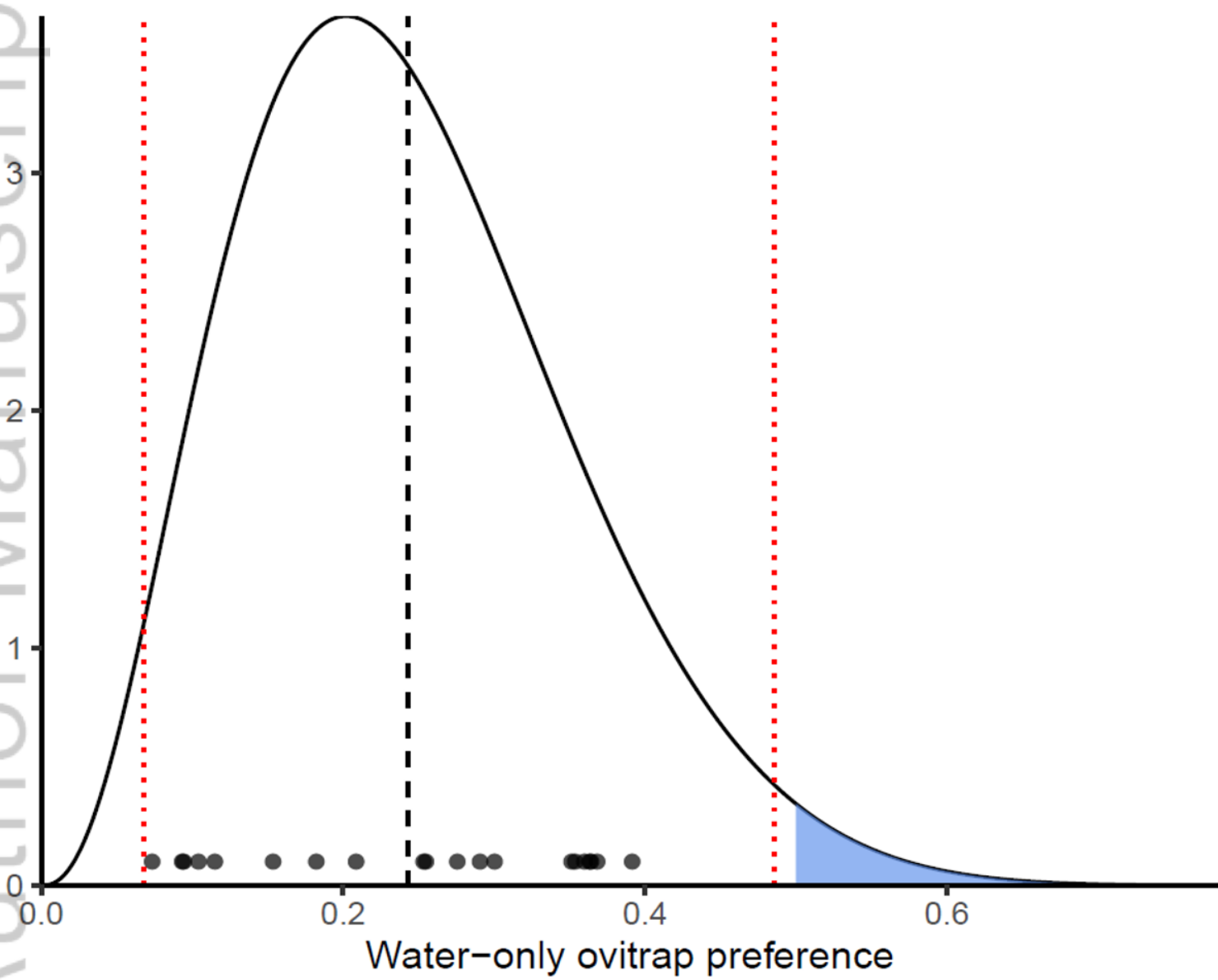
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Evaluation of large volume yeast interfering RNA lure-and-kill ovitraps for attraction and control of *Aedes* mosquitoes

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- Yeast interfering RNA insecticides, syt.427 and sema.460, were highly effective against *Aedes* larvae when deployed in 7.5 L ovitraps in controlled insectary studies, simulated field and semi-field studies.
- Oviposition and larval hatch rates were similar for individual *A. aegypti* females offered 7.5 L ovitraps baited with control yeast, syt.427 yeast, or sema.460 yeast in laboratory cage studies.
- Pair-wise field studies conducted in Trinidad, West Indies showed that *Aedes* females were significantly more likely to lay eggs in yeast-baited 10 L ovitraps than ovitraps containing water-only.